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Microbial Mineral Recovery

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Extracellular Polymers for Metal Binding

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Introduction

Capsular and slime exopolymers are elaborated by a wide variety of microorganisms in nature. These exopolymers serve as a buffer between the cell wall and the external environment. Under some circumstances the exopolymers serve as a barrier to harmful or toxic substances such as bacteriophage, antibiotics, and biocides. The polymers form a matrix that physically excludes large molecules, thus minimizing their contact with the cell wall and underlying cell components. Many exopolymers also carry a charge which promotes ionic and electrostatic binding of counterions. These interactions may, on the one hand, prevent excess quantities of charged molecules such as heavy metals from approaching the cell surface and yet facilitate the concentration of growth-promoting nutrients present at low concentrations in the surrounding environment for subsequent uptake by the cell.

The influence of metal ions on microorganisms is unique in that over a rather narrow concentration range, their status may change from that of an essential growth-promoting element to one that is toxic. The exopolymers therefore serve a critical function in regulating the exposure levels that the bacterial cells encounter in environments with widely fluctuating metal-ion concentrations. As one of the two main mechanisms of metal concentration by microbial cells, capsular polymers have evolved features which suggest they act as effective modulators of metal-ion concentration at the cell surface.

Tolerance of microorganisms to metals in important industrial processes has long been known to involve extracellular polymers. Early investigations on the interactions of metal ions with bacterial exopolymers arose from studies on the effects of metal wastes on sewage treatment microorganisms. The floc-forming strain 115 of *Zoogloea ramigera* was found to remove significantly more dissolved Co, Cu, Fe, and Ni from solution than a strain that did not produce a gelatinous polymeric matrix around the cells (Friedman and Dugan, 1968). Young cultures of the floc-forming strain exhibited a lower affinity for Zn than cultures which contained more exopolymer. Later, zinc binding by isolated exopolymer was confirmed.

Recent evidence suggests that interactions between capsular and slime exopolymers and certain metal ions contribute to corrosion reactions which have severe economic consequences (Geesey et al., 1986). Interestingly, the metal-recovery industry is interested in utilizing these biologically catalyzed reactions to improve metal-recovery efficiencies from mining operations and other metal-laden effluents. Thus, interactions between metals and these microbial exopolymers are of practical importance.

Ecological and geochemical processes are also influenced by the complexation reactions between metals and surface polymers of microbial cells. The importance of microorganisms in metal mobilization and diagenesis may well involve binding to exopolymers and other microbial metabolites. These processes, while far from being understood, are at least beginning to receive the attention they deserve.

In this chapter, biochemical and physicochemical features which promote interactions of microbial exopolymers with various metal ions in solution will be described. These interactions will be considered in terms of their influence on the exopolymers, the bacteria which produce the exopolymers, and the surrounding environment.

Nature of Bacterial Capsule and Slime

In this chapter, *exopolymer* refers to a macromolecule excreted outside the cell wall proper. In some cases, however, the exopolymer may be an extension of a cell wall component, such as the O-antigen portion of the lipopolysaccharide in the outer membrane of gram-negative bacteria. The microbial exopolymers with which most metal studies have been conducted are those which are commonly associated with capsules or slime. Capsules are invariably observed anchoring sessile microorganisms to each other, to other types of living cells, and to inanimate objects. They appear as the outermost envelope layer surrounding the cell. Capsules are elaborated by both gram-negative and gram-positive eubacteria, cyanobacteria, archaebacteria, and unicellular algae. Capsular exopolymers are firmly retained by the cell

and can withstand large shear forces (Pedersen et al., 1986). This association enables the cell to establish a stable orientation with respect to its neighbors and environment. Capsular exopolymers may extend from 0.1 to 10 μm or more from the cell surface into the surrounding environment, creating a buffer zone between the outer cell wall and the external environment. The widespread appearance of capsules among natural populations of microorganisms indicates that they are essential to survival.

In laboratory cultures, capsular polymers maintain a more transient association with the cell. In some cases, the exopolymers take the form of what is frequently referred to as "slime." Slime polymers slough from the cell into the surrounding medium. The extent to which this sloughing occurs in the natural environment remains to be determined. There is some evidence that overproduction of capsular polymers leads to sloughing. Production of slime may be controlled by environmental conditions. Different strains of the same microorganism may elaborate exopolymers that maintain different associations with the cell. In aquatic and industrial fields, the term *slime* is often used to refer to the biofilm which develops on submerged surfaces. The biofilm contains cells and their exopolymers and any adsorbed material from the bulk aqueous phase. The properties of slime will therefore differ, depending on how it is defined. In this chapter, the term *slime* will refer to the assemblage of polymeric material that is excreted from microbial cells, including dissolved material adsorbed from the culture solution but excluding the cells and their wall components.

Although most capsule and slime exopolymers described to date are composed of polysaccharide, considerable amounts of protein are frequently recovered in crude exopolymer preparations. Whether the proteins perform any function in this extracellular location remains to be determined. Other cell products such as DNA and RNA are also recovered with the capsule and slime exopolymers. It is likely that some or all of this material represents excretory or lysis products that have become trapped by the polysaccharide molecules. The extent to which they contribute to the overall interactions between the capsular polysaccharide component and other substances in the environment is a question that has not been widely studied.

The exopolysaccharide component of microbial exopolymers usually possesses a repeating sequence of from two to six sugar subunits. Some polymers such as alginic acid, however, possess blocks of repeating sugars in one segment of the polymer that are different from those in other segments. Many bacterial and algal capsular polymers are composed of acidic polysaccharides. The acidic properties are contributed primarily by free carboxyl groups of uronic acid subunits or pyruvylated sugars. In some instances, the exopolysaccharide exhibits

branching where two to five sugar subunits extend away from the polymer backbone. When both capsule and slime exopolymers are produced by the same bacterial culture, the chemical composition of their polysaccharides is usually similar (Cohen and Johnstone, 1964).

The subunit composition and structure determine the physical properties of the exopolymer. Most microbial exopolysaccharides are extremely hygroscopic. Greater than 99 percent of the volume of a hydrated exopolymer is contributed by water. Most capsules and slimes form a colloid or gel phase, depending on the nature of the surrounding environment. Aggregation of polymers occurs in the presence of divalent cations. Visible flocs may form under certain conditions. These flocs have been associated with "bulking" of activated sludge in the sewage treatment process. Some slime exopolymers possess unusual rheological properties that have been exploited for various industrial applications. The high viscosity of xanthan, a slime from the plant pathogen *Xanthomonas campestris* and alginic acid, a slime from several different species of bacteria and algae, has made these polymers useful as stabilizing agents in foods. In addition, metal cross-linked xanthan has been used as a fracturing gel in enhanced oil recovery (Menjivar, 1984). These characteristics make capsular and slime exopolymers unique among other naturally occurring biopolymers.

Properties of Microbial Exopolymers Relevant to Metal Binding

Many capsular and slime exopolymers of aquatic microorganisms act as polyanions under natural conditions. The capsules of most bacteria examined to date contain from 5 to 25 percent uronic acids (Sutherland, 1980). Some sugar subunits of capsules and slime possess ketal-linked pyruvate groups which generally contribute around 5 percent of the polymer weight (Sandford et al., 1977; Boyle and Reade, 1983). Several genera of enteric bacteria possess capsules containing sialic acids, which are derivatives of neuraminic acid (Dewitt and Rowe, 1961; Liu et al., 1971). The anionic character of polysaccharides containing any of the above components is conferred by the carboxylic acid moiety present. The presence of the carboxylic acid groups cause the polysaccharides to exhibit pK_a values between 3.5 and 5.0. At neutral to alkaline pH, the partially ionized carboxyl groups are available to interact with positively charged metal ions.

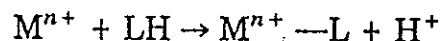
Polysaccharides also contain an abundance of hydroxyl groups which tend to interact with metal ions. The electronegative oxygen atoms of hydroxyl groups are likely to participate in metal interactions with anionic or neutral polysaccharides.

Some polysaccharides contain amino sugars or sugars with amide-linked functional groups. These nitrogen-containing functional groups are capable of reacting with some metals. To date, only a few exopolysaccharides have been found to contain amide or amine substitutions. Consequently, their importance in metal interactions with exopolymers remains to be determined. It is quite likely, however, that the metal-binding capacity contributed by the protein component of exopolymeric material involves these functional groups. Kihn et al. (1987) showed that Cu^{2+} was chelated by peptides and proteins extracted from walls of *Saccharomyces cerevisiae*. The binding sites were formed by an amide and a strongly complexing, amine-like ligand. In slightly acidic conditions, Cu^{2+} was bound by oxygen of the amide, whereas at basic pH, NHCO became deprotonated and the negatively charged nitrogen bound the metal.

Mechanisms of Metal Binding to Bacterial Exopolysaccharides

Several classes of polymeric molecules appear to participate in exopolymer-metal interactions. Protein and polysaccharide components from a firmly bound capsular polymer of a freshwater sediment bacterium exhibited similar binding capacities toward cupric ions (Mittelman and Geesey, 1985). Only the mechanisms of interaction between polysaccharides and metal ions, however, will be considered here.

The general interaction between a metal ion and an organic molecule may be described as an acid-base reaction:



where M^{n+} , the metal ion, or H^+ , the proton, represents the acid, and L, the organic molecule or ligand, represents the base. The release of protons by acidic polysaccharides during exposure to Cu ions has been reported in several instances (Zunino and Martin, 1977; Mittelman and Geesey, 1985). Binding of Cu ions resulted in a shift in the pK_a of a capsular polysaccharide from 4.90 to 4.05 (Mittelman and Geesey, 1985). These data suggest that competition exists between Cu ions and protons at the metal-binding site of acidic exopolymers.

Metal ions tend to form bonds with functional groups containing electron-donating atoms. Four types of interactions between metal ions and exopolymers have been described (Steiner et al., 1976). The two most important types were those that involved salt bridges with carboxyl groups on acidic polymers and those that involved weak electrostatic bonds with hydroxyl groups on neutral polymers. In

polysaccharides containing uronic acids or pyruvate groups, lone-pair electrons on oxygen atoms of carboxyl groups have a strong tendency to interact with charge-compensating metal ions. Oxygen atoms in the ether bonds and hydroxyl groups of sugar subunits act as weak electron donors in both acidic and neutral polysaccharides (Martell, 1971). The "S-type" isotherm produced by complexes between metal ions and the capsule of *Z. ramigera* 115 was proposed to involve primarily hydroxyl groups of the glucose subunits even though the polysaccharide contains free carboxyl groups on the ketal-linked pyruvate residue (Brown and Lester, 1979). This type of metal binding is believed to be the most important mechanism of metal removal in activated sludge.

Manzini et al. (1984) emphasized the importance of carboxyl groups in cupric ion binding. They suggested that an intimate interaction exists between Cu ions and carboxyl groups on acidic polysaccharides. Cupric ion binding to a carboxyl group is electrovalent. In the case of polyuronates, the mode and extent of the interaction are believed to depend on several factors: the nature of the component sugars and their relative distribution in the chain, the magnitude of the overall electrostatic field, and the ratios of Cu to polymer and of Cu to simple supporting electrolyte.

The modes of interaction change as the ratio, R , of total metal ions to COO^- groups varies. As the ratio decreases from a maximum of 0.50, the average number of uronate groups in alginic acid that interact with each Cu ion increases to more than two. Under optimum conditions 85 percent of the COO^- groups can interact with Cu^{2+} . The remaining 15 percent are inaccessible for complex formation. This is due to some extent to competition between Cu ions and counterions such as Na^+ for the charged site on the polymer molecule.

Linear charge density on acidic polymers is believed to influence metal interactions. The stability of the interaction has been shown to increase with increasing charge density (Mathews, 1960). A polysaccharide exhibits a stronger interaction with metal ions when it exists in a gel state (high charge density) than when it exists in solution (lower charge density). Ionic bonds between a single polyvalent cation and two anionic groups on separate polymer chains are believed to contribute to the stability of the gels. This idea is supported by evidence that Cu^{2+} complexes with two uronate groups in the case of alginic acid (Manzini et al., 1984). Charge transfer occurs from the negative charge on the oxygen atom of the COO^- to the Cu^{2+} ion.

Charge transfer is very sensitive to the stereochemical environment. Often, the ionic radius of a metal determines whether complexation will occur. For uncharged polysaccharides, affinity for a metal usually decreases with increasing ionic radius of the metal. In contrast, carboxylated polysaccharides often exhibit preferential bind-

ing to cations with large ionic radii. Affinity of alginic acid for the transition metals decreases in the order $\text{Cu} > \text{Ni}$, $\text{Co} > \text{Zn} > \text{Mn}$ which roughly follows the Irving-Williams order of decreasing complex stability.

With few exceptions, carboxylated polyanions exhibit higher selectivity for transition metals than do the alkali- and alkaline-earth metals. In some instances, however, the affinity of Mg fluctuates relative to other cations in different anionic polysaccharides. In addition, selectivity coefficients may be different, depending on whether the polymer exists in solution or in a gel state.

Volume change on dissociation of ionizable groups along alginic acid chains is simply a linear function of the degree of ionization (Paoletti et al., 1981). Dissociation occurs without any interference among the hydration spheres of the ionizable (COO^-) groups. The ionization behavior of alginate does not depend on whether the counterion is Na^+ or K^+ . Volume change is related to a change in the extent of hydration of the cation. A net volume increase occurs during the binding of Cu^{2+} to carboxylate groups on sodium alginate.

Cu ions in solution displace Ca ions bound to alginic acid. A high selectivity for Cu over Ca is a characteristic feature of carboxylated polyanions, in general. The binding mechanism that is responsible for the higher affinity for Cu than Ca appears to be independent of free hydroxyl groups in the polymer and of the steric arrangement of the carboxyl groups (Haug and Smidsrod, 1970).

Differences in degree of ion hydration and hence the distance of closest approach between the cation and the site of interaction on the ligand may influence cation selectivity. Energy required for ion dehydration must also be taken into account. Metal ions in solution are always fully coordinated or solvated by water molecules. When a metal ion becomes complexed by an organic ligand, there is a displacement and reordering of water molecules: the number of water molecules displaced depends on the dimension and charge of the metal ion and on the size of the coordinated electron-donating group of the ligand. It was proposed that Cu ions have a larger ordering effect on the water molecules than do Ca ions, and that the displacement of Ca by Cu on the polymer should be favored by the relatively large positive entropic change (Haug and Smidsrod, 1970). The reaction has a positive enthalpy of formation and is entirely entropy-driven. In alginic acid and hyaluronic acid, cations and most water molecules have well-defined positions and may determine gross configuration of the polymer chain.

Paoletti et al. (1981) suggest that the interaction between COO^- groups on alginic acid and Cu^{2+} ions loosens a relatively large number of water molecules from the solvation shells of both ionic species, thus producing a net gain of molecular degrees of freedom in the system.

This occurs when the ratio of moles of Cu^{2+} to equivalents of alginate in solution is less than 0.4. At higher ratios, gel formation occurs.

Order within the liquid subphase may also depend on the chemical properties of the capsule. In the case of M41 capsular polysaccharide of *Escherichia coli*, only the polyanions are ordered and the cations and water molecules behave like a liquid filling the spaces between polymer molecules (Moorhouse et al., 1977).

Differences in selectivity can be due to variations in stereochemistry of the sugar subunits comprising the polysaccharide. For the exchange reactions Ca-Mg, Ca-Sr, Sr-Mg, and Co-Ca, the mannuronic acid residues in alginic acid (gel) have selectivity coefficients of unity (Smidsrod and Haug, 1968). However, selectivity coefficients for exchange reactions Sr-Mg and Co-Ca for guluronic acid residues were 150 and 0.17, respectively. The high selectivity of guluronic acid residues in the alkaline-earth exchange reactions appears to depend on the existence of alginate in an insoluble gel state. Both residues demonstrate a selectivity of Cu over Ca.

The stability and selectivity of complexes between alkaline-earth ions and alginates are believed to be influenced by hydroxyl groups (Cozzi et al., 1969). Differences in affinity among these cations for polymannuronic, polygalacturonic, and polyguluronic acid were attributed to variations in the positioning of hydroxyl groups (Haug and Smidsrod, 1970). It appears that the spacing of coordinating oxygen atoms in the polyuronate molecule determine which cations will bind as well as their stability. The availability of two hydroxyl groups on the uronate subunit is more stabilizing than one. In general, the more coordination bonds that are formed between the metal and the ligand, the greater is the stability of the complex. The formation of stable complexes between metal ions and anionic polysaccharides usually involves both electrovalent (nonionic) and coordinate covalent bonding.

A series of cavities that offer 4-oxygen coordination with Ca ions exists in segments of alginic acid which contain repeating sequences of guluronic acid (Rees, 1972; Kohn, 1975). Cooperative binding of the guluronic-acid segments with functional groups on other adjacent polymer chains is necessary for cavity formation. Fewer cavities exist in segments which contain repeating sequences of galacturonic. These cavities offer only three oxygen atoms for coordination. Only shallow cavities are formed with polymannuronic acid. The depth of the cavity and the number of available coordinating oxygen atoms are believed to determine the degree of selectivity and stability for Ca and Sr ions. Steric fit therefore seems to be important and supports the evidence that ionic radius of the metal frequently determines whether complexation will occur.

Using the linear charge model for the enthalpy of dilution of

polyelectrolyte solutions, a study was performed by Pass and Hales (1981) to investigate the effects of the cation on the enthalpy of dilution of alkali-metal salts of alginate. They proposed that the enthalpy change that occurs during the interaction between polyelectrolytes and counterions may be considered in terms of at least three possible effects: enthalpy changes occurring on dilution, enthalpy changes arising from interactions between the ion atmosphere of the polyelectrolyte and the counterion, and enthalpy changes involving the site-bound counterions.

The enthalpy of dilution depends on the charge density on the polyelectrolyte. The enthalpy is increasingly exothermic as the charge density decreases and as the size of the counterion decreases ($K < Na < Li$). The enthalpy of dilution of carboxylated polyanions shows a dependence on the counterion: $Li > Na > K$.

The enthalpy of interaction of a polysaccharide with a cation is influenced by the concentration of the cation. The enthalpy of mixing the salt form of a carboxylated polysaccharide with increasing concentrations of a chloride salt of the same cation becomes more endothermic as the concentration of the salt increases (Pass and Hales, 1981). The enthalpy change depends on the nature of the cation.

Mixing alginate of one cation salt with the salt of another cation alters the concentration of the counterions in the ion atmosphere and at the binding site (Pass and Hales, 1981). Enthalpy changes become more endothermic when an alkali-metal alginate is mixed with an alkali-metal chloride containing a lighter counterion. The replacement of a site-bound cation by a heavier cation is an exothermic process.

The equilibrium reactions between the alkali-metal cations and carboxylated polyanions exhibit affinities in which $Li > Na > K$. These observations, along with the enthalpy data, suggest that the endothermic replacement of a site-bound cation with a lighter cation is accompanied by an increase in total entropy. This would then be consistent with the observed volume changes which are related to changes in the extent of hydration of the counterion. Exchange of a cation such as Li from solution with a site-bound cation such as Na results in a positive enthalpy change and an increase in entropy when there is a net loss of bound water molecules. Metal-ion complex formation with carboxylated ligands has a positive enthalpy of formation and is also an entropy-driven process.

The bond strength between the site-bound counterion and the carboxylated polyanion is not controlled by the nature of the counterion (Paoletti et al., 1981). Binding will occur as long as there is a greater release of water molecules. Thus, binding affinity is related

more to entropy changes than to bond strength, which is related to enthalpy changes.

For interactions between counterions and sulfated polyanions, less dehydration of the counterions occurs than that observed with carboxylated polyanions. The sequence of volume changes is reversed, so $\text{Li} < \text{Na} < \text{K}$. The enthalpy change and the equilibrium, free-energy change follow the same sequence. The sequence of enthalpy changes indicates that the bond strength between a site-bound alkali metal and a sulfated polyanion such as dextran sulfate or carageenan increases with increasing cation size.

Mo binding by extracellular slimes of rhizosphere bacteria was investigated by Tan and Loutit (1976). Greater than 90 percent of the Mo associated with cells of a *Pseudomonas* sp. was bound to the slime. Mo was mainly bound by a passive process to the uronic acid-containing subunits of the slime layer. The slime from *Pseudomonas aeruginosa* was reported to bind Mo through the glucuronic-acid subunit (Stojkovski et al., 1986). Cu was also bound by this subunit. Glucuronic acid contributed 32 percent of the total slime weight. Complexation occurred through the two oxygen atoms of the carboxyl group and the oxygen atom of the hydroxyl groups of C-3 and C-4 of the uronic-acid molecule. As much as 97 percent of the total Mo concentrated by the cells was bound to the slime.

Enzymes were used by Flatau et al. (1985) to identify sites of Cd binding by cell surface polymers of a marine pseudomonad. Of the enzymes tested, pectinase was found to liberate the greatest amount of Cd-binding material from cell envelopes, suggesting that galacturonic-acid residues were the most important complexing agent. Release of lesser amounts of Cd was achieved by treatment of the envelopes with cellulase. It was suggested that neutral sugars also contributed to the binding reaction.

A large number of metals have been reported to successfully cross-link polysaccharides. Carboxyl groups are active cross-linking sites on acidic polysaccharides while for neutral polysaccharides, any number of hydroxyl groups should be able to serve as cross-linking sites. There are several processes which compete with the cross-linking reaction. One process is the hydrolysis of the metal ion. In aqueous solutions, hydrated metal ions behave as acids and may hydrolyze by releasing one or more protons from coordinated water molecules (Menjivar, 1984). The extent of hydrolysis depends on the polarizing properties of the metal ion and the pH of the solution. At low pH, protonation of the ligand competes with the cross-linking reaction. The optimum pH range for the gelation of a polymer with a metal will depend on the acid-base properties of the ligands on the polymer and on the hydrolysis properties of the metal.

Purified preparations of the floc polysaccharide isolated from *Zoogloea* spp. bound different amounts of alkaline-earth and transition-metal ions. The relative affinity of the cell-floc material for the transition-metal ions was determined to be $\text{Fe} > \text{Cu} > \text{Co} > \text{Ni}$ (Dugan and Pickrum, 1972). Approximately 25 percent of the floc weight was contributed by bound metal ions. The combined polysaccharide species purified from crude floc produced by cells of *Z. ramigera* 115 was reported to bind $0.25 \mu\text{mol Fe}^{3+}$ per milligram polysaccharide (Ikeda et al., 1982). Dugan proposed that metal binding occurred through an exchange reaction with polymer-bound water molecules and that the removal of water resulted in the observed flocculation that occurred in the presence of metals.

Flocculation of solutions containing either Cu or Cd with floc-producing strains of *Z. ramigera* 115 reduced the dissolved metal concentration to less than 0.1 and 0.0005 g/L, respectively. The complexed metals could be released from the flocs at pH 3 to 4. These results suggest that the metals were complexed by carboxyl groups on the polymers via ionic bonding.

Metal uptake was compared between capsule-producing and noncapsulated strains of *Klebsiella* (*Enterobacter*) *aerogenes* (Rudd et al., 1983). Of the metals tested (Cu, Cd, Ni, Mn, Co), all but Ni were accumulated to a greater extent by the encapsulated strain. Six- to 10-fold more Cu and Cd were concentrated by the capsular polymers than was taken up by the cells. A shift from cellular uptake to extracellular binding of these metals was observed in chemostat cultures as the dilution rate was decreased. The extent of metal removal from solution by the capsulated strain followed the order $\text{Cu} > \text{Cd} > \text{Co} > \text{Mn} > \text{Ni}$. This sequence of affinity is similar to that displayed by *Z. ramigera* 115 flocs and to laboratory-scale activated sludge biomass.

The relative affinity of isolated extracellular polymer from *E. aerogenes* for metal ions differed slightly from that exhibited by encapsulated cells. Freundlich isotherms indicated that the relative affinities of metals for sites on the exopolymer material followed the sequence $\text{Cd} > \text{Co} > \text{Ni} > \text{Mn}$ (Brown and Lester, 1982). At 0.1 mg/L free Co, Cd, or Ni, the polymer was close to saturation at approximately 1 mg metal-bound per gram polymer.

The sheath material of a *Microcystis* species of cyanobacteria concentrated Fe and Mn by factors of 10^4 and 10^5 , respectively (Amemiya and Nakayama, 1984). The sheath material contained fivefold higher concentrations of metals than whole cells. Based on Freundlich isotherms, the affinity of the sheath for metals followed the series $\text{Fe} > \text{Zn} > \text{Mn} > \text{Cu}, \text{Ni}$. The sheath contained 35 to 47 percent carbohydrate, 12 to 15 percent uronic acids, and 18 to 24 percent protein.

On the basis of these and other studies conducted to date, it may be

concluded that bacterial exopolymers exhibit selectivity for metal ions. Oxygen atoms of carboxyl and hydroxyl groups participated in metal-ion binding by bacterial exopolysaccharides. The selectivity for one cation over another is determined by a variety of factors involving the properties of both the cation and the polysaccharide. Some features of polysaccharides which promote interactions with metal ions are clearly distinct from those exhibited by classical polyelectrolytes.

Binding Constants for Exopolymer-Metal Complexes

The type of quantitative information needed to make valid comparisons of affinities of different exopolymers for metal ions is often not available. As a result, it is difficult to compare binding strength, competition between different metals, and binding site density for those exopolymers that have been shown to interact with metal ions. Stabilities of complexes between metal ions and ligand-binding sites are based on the equilibrium constants for their formation. To date, only a few stability constants have been determined for bacterial exopolymer-metal complexes.

Rudd et al. (1984) obtained $\log K_i$ values for Cu, Cd, Co, and Ni complexes with capsular material from *E. aerogenes* Type 64. The constants were determined at one pH (6.3), one polymer concentration, and at metal concentrations ranging from $0.2 \mu\text{M}$ to 0.1 mM . An indirect correlation was observed between the stability constant and the number of binding sites on the polymer molecule. The number of binding sites per molecule ($1.7 \times 10^6 \text{ MW}$) ranged from 51 for Cd to 7 for Cu. The complexation capacity (mol/g dry weight) was 4×10^{-6} and 3×10^{-5} for Cu and Cd, respectively. Only one type of binding site was identified for each metal. The binding sites exhibited some specificity for the different metal ions. Only Co was reduced in overall binding when the polymer was exposed to combinations of metals. These observations were contradicted, however, by evidence indicating that the polymer possessed only two distinct types of binding sites. The capsule contained neutral hexose sugars, uronic acids, and sugars substituted with acetate and pyruvate.

The conditional stability constants and maximum binding activity (MBA) of Cu binding sites on protein and polysaccharide components of the capsule from a freshwater sediment bacterium (FRI) were compared by Mittelman and Geesey (1985). As the protein(s) component was removed, binding sites were lost, but the stability constant remained relatively constant. A highly purified polysaccharide fraction exhibited an MBA of 253 nmol Cu per milligram carbohydrate. On a dry-weight basis, humic acid possessed an MBA 20 times higher than

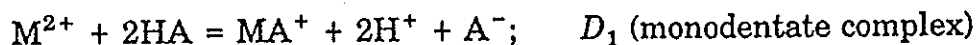
that of the crude exopolymer. Their stability constants were comparable, however. Infrared spectroscopic analysis indicated that Cu binding involved a carboxyl residue on the polymer molecule (Geesey et al., 1988). Experiments conducted at Cu concentrations, pH, and temperatures of sediments from which the bacterium was isolated indicated that the exopolymer was capable of binding the metal under natural conditions. In view of these results, it is likely that bacterial exopolymers compete with other metal complexing agents for metal ions in nature.

Intrinsic Stability Constants

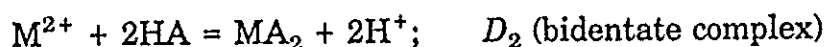
Although thermodynamic binding constants for metal-ligand complexes are used to compare affinities of different metals for a particular ligand and for different ligands, the values determined are conditional in nature when they are based on Langmuir's model and Scatchard's plotting procedure. Stability constants obtained by these methods vary with environmental conditions such as pH, ionic strength, polymer concentration, and temperature. The determination of intrinsic stability constants and binding-site densities, which are independent of reaction conditions, requires an accurate estimation of the activities of interacting chemical species at the site of complex formation. Such information has been difficult to obtain in the case of anionic polysaccharides.

The electrostatic field component created by the negatively charged reaction sites of anionic polysaccharides produces a higher concentration of counterions in the aqueous region immediately adjacent to the polymer chain (polymer subphase) than that found in the bulk aqueous phase. The activities of the counterions in the polymer subphase can be estimated, however, from the measured activities in the bulk solution by applying Donnan equilibrium theory (Geesey and Jang, 1989). Base titration of the polymer at different ionic strengths provides a partition coefficient $10^{n\Delta pK}$ (where n = valence of the counterion and $\Delta pK = pK_{HA}^{app} - pK_{HA}^{int}$, where superscripts app and int refer to the apparent and intrinsic acid dissociation constants, respectively), which describes the distribution of the counterion between the polymer subphase and the bulk aqueous phase. A numerical procedure based on this multiphase model, the colligative properties of the polymer solution, and Donnan equilibrium theory may then be used to estimate the volume of the polymer. Dividing the moles of ionizable ligands by the volume of the polymer subphase (instead of the volume of the bulk liquid) provided an accurate description of the concentration of ligands that were available for Cu binding by alginic acid (Jang et al., 1989).

Since polyvalent metal ions appear to bind more than one type of ligand on the polysaccharide molecule, unique stability constants exist for each type of interaction. This has been shown to be the case for Cu binding by alginic acid (Geesey and Jang, 1989). If titration of the polymer in the presence of metal yields data that do not provide the same stability constant for the reaction



at different ionic strengths, fractions of ligands ionized, and polymer concentrations, then it is likely that a portion of the bound metal ions formed a bidentate complex:



D_1 and D_2 are obtained from the slope and Y-intercept of plots of D'_1 versus A^-/V_p and D'_2 versus V_p/A^- , where

$$D'_1 = \frac{(H^+)^2[M_{sb}][A^-]}{(M^{2+})[HA]^2}$$

In this equation, M_{sb} ($MA^+ + MA_2$) is the moles of site-bound metal in the polymer subphase and replaces MA^+ when the apparent distribution coefficient D'_1 is defined; V_p is the polymer subphase volume, and the other symbols have their usual meanings. The parentheses refer to activity, and the brackets refer to a grouping of mathematical terms. Similarly, if MA_2 is replaced by M_{sb} , another distribution coefficient, D'_2 , may be defined as

$$D'_2 = \frac{(H^+)^2[M_{sb}]V_p}{(M^{2+})[HA]^2}$$

D_1 and D_2 are related to D'_1 by the expression

$$D'_1 = D_1 + \left[\frac{D_2 A^-}{V_p} \right]$$

and to D'_2 by the expression

$$D'_2 = D_2 + \left[\frac{D_1 V_p}{A^-} \right]$$

The intrinsic stability constants $\beta_{MA^+}^{int}$ and $\beta_{MA_2}^{int}$ of monodentate and bidentate complexes, respectively, formed between the metal and ligands on the polymer are obtained by dividing D_1 or D_2 by the square of the inverse of the acid dissociation constant (K_{HA}). By this approach, stability constants for the monodentate complex and

bidentate complex between alginic acid and Cu^{2+} ions were found to be 832 and 67, respectively (Jang et al., 1989).

The proportion of metal bound to the polymer via monodentate and bidentate complexes can be calculated from the expression

$$\frac{\text{Moles monodentate complex}}{\text{Moles bidentate complex}} = \frac{D_1^{\text{int}}}{D_2^{\text{int}}} \times \frac{A^-}{V_p} = \frac{\beta_1^{\text{int}}}{\beta_2^{\text{int}}} \times \frac{A^-}{V_p}$$

From these determinations it was possible to compare the values obtained for the intrinsic stability constant $\beta_{\text{MA}}^{\text{int}}$ and the conditional stability constant $\beta_{\text{MA}}^{\text{app}}$. For the monodentate complex constant the value for $\beta_{\text{MA}}^{\text{app}}$ is higher than the $\beta_{\text{MA}}^{\text{int}}$ by the factor $10^{2\Delta\text{pK}}$, and for the bidentate complex $\beta_{\text{MA}}^{\text{app}}$ is higher than $\beta_{\text{MA}}^{\text{int}}$ by the factor $10^{2\Delta\text{pK}} V_s/V_p$, where subscripts *s* and *p* refer to the volumes of the bulk liquid phase and polymer subphase, respectively. Both the partition coefficient $10^{2\Delta\text{pK}}$ and the volume of the polymer subphase vary with environmental conditions as well as the degree of polymer ionization and extent of metal binding to the polymer.

Influence of Environmental Conditions on Properties of Exopolysaccharides Relevant to Metal Interactions

Bacterial exopolymers are highly influenced by growth conditions in the environment. Metal adsorption by crude floc from *Z. ramigera* 115 was influenced by pH. A maximum of 3 mmol Cu was adsorbed per gram dry weight at pH 6 to 7 and a maximum of 1.8 mmol Cd per gram dry weight at pH 8.0 (Norberg and Persson, 1984). The binding capacity of the floc was not influenced by changes in ionic strength. The capacity of the floc to bind iron was enhanced by the presence of phosphate (Dugan and Pickrum, 1972).

Tait et al. (1986) showed that exopolysaccharide synthesis by *X. campestris* decreased as the growth rate decreased. Exopolymer isolated from chemostat cultures at high dilution rates had higher acetyl content and lower pyruvate content than that recovered from cultures at low dilution rates. Uhlinger and White (1983) provided evidence that exopolymer formation by *Pseudomonas atlantica* was enhanced by an increase in available surface area. In estuarine sediments, the exopolymer contributed a carbon content at least as great as the cells themselves. The greatest accumulation of exopolymer occurred in the stationary phase when cells were stressed. The composition of the *P. atlantica* exopolymer changed during the growth cycle. The proportion and amount of uronic acids increased relative to neutral hexoses as the rate of exopolymer synthesis increased. In other studies, the quantity

and composition of slime polysaccharide by *P. atlantica* was found to vary with dilution rate in chemostat cultures (Gordon et al., 1988). At low dilution rates (0.005 h^{-1}) the uronic acid content was lower than at high dilution rates (0.01 to 0.04 h^{-1}). Galacturonic-acid content increased with increasing dilution rate. Glucuronic acid appeared only at the highest dilution rate. These results indicate that the properties of bacterial exopolysaccharides are quite variable and appear to be determined by conditions of the surrounding environment. It is therefore likely that the metal-binding characteristics of the exopolymers also depend on the environmental conditions.

Differences in the intrinsic stability constants for Cu binding were observed for slime polymers from chemostat cultures of *P. atlantica* grown at different dilution rates (unpublished results). The highest stability constants for both mono- and bidentate complexes occurred at a dilution rate of 0.015 h^{-1} . At this dilution rate, the exopolymer exhibited the highest carbohydrate content ($400 \mu\text{g}/\text{mg}$ dry-weight polymer). These results suggest that the carbohydrate component of the exopolymer exhibits the greatest stability with respect to cupric ions.

Influence of Metals on Properties of Microbial Exopolysaccharides

Metals are known to influence the production of capsule and slime polysaccharides. Metal and metalloid ions serve as cofactors in polysaccharide synthesis. Exopolysaccharide synthesis by *E. aerogenes* is stimulated by Mg, K, and Ca ions (Wilkinson and Stark, 1956). Polysaccharide production in *Chromobacterium violaceum* is enhanced in the presence of Fe and Ca ions (Corpe, 1964). The yield and form of exopolymer produced by a coryneform bacterium isolated from Cr-polluted sediments were influenced by the amount of Cr in the culture medium (Bremer and Loutit, 1986a). Exopolysaccharide production increased as more Cr(III) was added to the medium.

The presence of metals in the culture medium has been shown to influence the properties of the exopolysaccharide synthesized during bacterial growth. The morphology of the capsule of *Azotobacter chroococcum* changes as a result of metal availability in the growth medium (Ferala et al., 1986). Under iron- or molybdenum-sufficient conditions, the capsule is condensed, whereas under deficient conditions the capsule appears more diffuse and extensive. It is not yet known whether these morphological differences reflect direct interaction between the metal and the polymers or whether the elevated metal concentrations affect polymer subunit chemistry at the level of synthesis.

Interactions with certain metal ions cause many capsular and slime exopolymers to flocculate. Metals that exist as free cations or as hydroxides promote flocculation and rapid settlement of bacterial

exopolymers in solution (Brierley and Lanza, 1985). At very high metal concentrations (0.2 M), extracellular polymers from *P. atlantica* formed a water-insoluble precipitate with Fe, Cu, and Pb (Corpe, 1975). Binding of metal ions causes some slime polymers to condense into gels. Carboxylated polymers often form gels in the presence of Cu ions (Brown and Lester, 1979). Controlled gel formation of alginates by Ca ions has created new applications for this polysaccharide in a variety of commercial enterprises.

Coprecipitation is frequently observed when bacterial exopolysaccharides come in contact with metal ions. Purified polysaccharide from *Zoogloea* sp. flocculated in the presence of several metals (Ikeda et al., 1982). Maximum polymer precipitation occurred in the presence of 0.5-mM ferric-iron solution. Ferrous iron was about 50 percent as effective in this regard as ferric iron. Since binding of metal ions to this exopolymer promoted flocculation, the reaction was proposed as a means of removing metal ions in a concentrated form from acid mine water (Dugan, 1970).

The capacity of capsular polymers to bind metals depends on the form in which the polymer exists. Rudd et al. (1984) compared the metal-binding capacity of colloidal and soluble forms of capsular material recovered from *Klebsiella (Enterobacter) aerogenes*. They determined that approximately five times more Ni complexed with the soluble form than with the colloidal form. Cu, Cd, Mn, and Co were also complexed to a greater extent by the soluble form of the polymer. However, estimation of the metal-binding capacity of polymer in these studies did not employ the rigorous approach required for polymers that form a colloid or gel phase. Consequently, these results should be viewed with caution.

Removal of Ca from culture medium leads to an increase in the relative concentration of mannuronic acid in alginic acid synthesized by *Azotobacter vinelandii* (Couperwhite and McCallum, 1974; Annison and Couperwhite, 1984). Appanna and Preston (1987) showed that exopolysaccharide production by an arctic *Rhizobium* was affected by amendments of Mn to the culture medium. Additions of 400 μM Mn^{2+} as $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ resulted in a 14 percent increase in carbohydrate as glucose, a 20 percent decrease in uronic acid, and a reduction in viscosity in the exopolymer produced by the cells. These results demonstrate that the metal-ion makeup of the surrounding environment clearly affects the properties of bacterial exopolysaccharides.

Ramifications of Exopolymer-Metal Interactions

Metal tolerance

Under laboratory conditions, bacteria that elaborate exopolymers are often more tolerant to heavy metals than mutants that are unable to

produce protective capsules and slime. Survival of capsule-producing strains of *E. aerogenes* in the presence of 10 $\mu\text{g/mL}$ Cu^{2+} or Cd^{2+} was significantly greater than in mutants which lost their capsule-producing ability (Bitton and Friehofer, 1978). Furthermore, capsular material isolated from the wild-type strain enhanced the survival of the mutant strain when added to a metal-containing suspension of the latter. The capsular polymer was shown to bind both Cu and Cd ions. The capsular polymer was also demonstrated to provide protection against Cd (Bauda and Block, 1985).

Nonmucoid variants of a coryneform bacterium isolated from Cr-contaminated sediments were less tolerant to Cr(III) than a mucoid wild-type strain (Aislabie and Loutit, 1986). The mucoid strain accumulated more Cr(III) than the nonmucoid strain. Greater than 80 percent of the metal was complexed to the exopolymer. These and other studies conducted to date relate the tolerance to a reduction in free metal concentration at the cell surface.

Bioaccumulation

Complexation of metals by microbial biofilms has been suggested to play an important role in controlling toxic metal concentrations in the bulk water of aquatic environments. Bottom sediments contain high densities of sessile bacteria that are attached to particle surfaces via their capsular exopolymers. The polymers extend over the surface of and between particles to form a biofilm and are optimally oriented to interact with metal ions in solution that approach the particles.

Since sessile bacteria contribute a significant portion of the total living biomass in sediments and, in addition, represent a source of high nutritive value, they are a preferred food source for many benthic invertebrates and vertebrates. Bacterial exopolymers were suggested to be a source of nutrition for the deposit feeding clam *Macoma balthica* (Harvey and Luoma, 1984). Sediment-bound Cd, Zn, and Ag were taken up by the clam during ingestion of the bacterial exopolymer material (Harvey and Luoma, 1985). Patrick and Loutit (1978) demonstrated that metals, initially concentrated by bacteria and passed on through oligochaete worms, eventually accumulated in fish. In another study, it was shown that the Cr content of the snail *Amphibola*, which was fed Cr-complexed bacterial polysaccharide, contained threefold higher levels of the metal than snails fed polysaccharide containing very low levels of Cr (Bremer and Loutit, 1986b). Examination of body parts demonstrated that the bulk of the Cr was associated with internal tissues with lesser amounts adsorbed to the shell of the snail. It was further shown that polysaccharide with bound Cr was degraded more slowly than Cr-free polysaccharide by the heterotrophic sediment bacteria. These studies suggest that

detritivores are capable of accumulating exopolymer-bound metals during their ingestion of the bacteria. The capsular and slime exopolymers excreted by sessile bacteria therefore provide a means of metal entry into the aquatic food chain.

Capsular polymers are also used by epiphytic bacteria to attach to plant material. Montgomery and Price (1979) indicated that the largest net uptake of metals from a source of sewage pollution occurred in the epilithic microbial community of a model turtle grass-mangrove ecosystem. In another study, bacterial epiphytes were shown to be a major factor in contributing to the total Cr, Cu, Fe, Pb, and Zn of aquatic plants (Patrick and Loutit, 1977). Binding of Mo by extracellular polysaccharides produced by the epiphytic bacteria resulted in a reduction in the amount of metal that entered the plant (Lee and Loutit, 1977). Thus, the capsular polymers of bacteria can either enhance or inhibit metal uptake by higher life forms.

Metal deposition/oxidation

The acidic polysaccharides and protein material which are present in the sheaths of the *Sphaerotilus-Leptothrix* group have been suggested to serve as the matrix for ferromanganese-oxide deposition (Ghiorse, 1986). The sheath appears as an extensive fibrillar network of fine threads not unlike capsular polymers of other bacteria when viewed by transmission electron microscopy. Although the chemical composition of bacterial sheaths has not been well-characterized, Romano and Peloquin (1963) found that the sheath of *Sphaerotilus natans* contained carbohydrate (36 percent), amino sugars (11 percent), protein (27 percent), and lipid (5 percent). Reaction of the sheath with ruthenium red suggests that the sheath of *Leptothrix discophora* possesses acidic groups (Ghiorse, 1986). A variant of a strain of *L. discophora*, which produced no sheath, still oxidized Mn^{2+} (Adams and Ghiorse, 1986). Thus, it appears that the Mn-precipitating action of the sheath is not necessarily related to Mn oxidation by this bacterium.

Macroparticles suspended in seawater below depths of 100 m contain exopolymer-producing bacteria that precipitate Mn and Fe (Cowen, 1983). Small variable amounts of Ca, Ba, Cu, and Zn were also bound by the halo of exopolymer material. The capsular metal deposits appear to contribute a major portion of the weakly bound fraction of the particulate Fe flux (Cowen and Silver, 1984). It was postulated that the Fe- and Mn-depositing bacteria associated with suspended macroparticles constitute a locally significant influence on Fe and Mn geochemistry.

Corrosion of metals

Recent studies have implicated bacterial exopolymers and other acidic polysaccharides in the corrosion of metals. White et al. (1986) showed

that the corrosion of 304 stainless steel in seawater was accelerated by slime formation and biofilm development over the metal surface. The corrosion induced by two *Vibrio* strains was reversed by removal of their exopolymer from the surface. Nonhomogeneous distribution of microbes and their exopolymers on the metal surface was believed to create sites of different cathodic activity which accelerated the observed corrosion. For this reason, most microbially influenced corrosion results in the formation of irregularly spaced pits.

Iwaoka et al. (1986) showed that gum arabic, a uronic-acid-containing polysaccharide from the acacia plant, was capable of adsorbing to metallic Cu films. Approximately 50 percent of the polymer remained adsorbed to the film after gentle rinsing with water. Subsequent to polymer adsorption, a decrease occurred in the thickness of the metal film as determined by attenuated total reflectance with Fourier-transform infrared spectrometry. Since no change in film thickness occurred in the presence of water alone, it was proposed that the adsorbed polysaccharide promoted ionization of the metal. Subsequent studies demonstrated that the ionized metal was bound to the polysaccharide (Jolley et al., 1989).

Different polysaccharides, including the capsule from a sediment bacterial isolate, caused different rates of metallic Cu dissolution (Geesey et al., 1987). The Cu was ionized in either cupric or cuprous form, depending on the polysaccharide that adsorbed to the metal surface. In the presence of gum arabic, the metal was oxidized to Cu^{2+} , whereas in the presence of culture menstruum containing slime excreted by a marine bacterial isolate referred to as LST, the Cu was oxidized to Cu^+ (Jolley et al., 1989). No dissolution or ionization of the Cu film was observed, however, when it was exposed to an aqueous suspension of a slime polysaccharide from *P. atlantica* over the 40-h test period (Geesey et al., 1988). All of the polysaccharides tested, including that from *P. atlantica*, were capable of binding Cu ions (unpublished results). These results demonstrate that the exopolysaccharides elaborated by bacteria have the capacity to adsorb to metal surfaces and bind metal ions to the extent that metal ionization is enhanced. This reaction may involve the liberation of protons and a reduction in pH at the metal surface (Geesey et al., 1986). Thus, the interactions between Cu ions and bacterial exopolymers provide a mechanism whereby bacterial biofilms can promote corrosion of Cu and perhaps other metal surfaces under aerobic conditions without intervention of the bacterial cell and its associated activities. Whether such a mechanism contributes to the commonly observed pitting of Cu and Cu alloy tubing used in industrial heat exchanges and water distribution systems remains to be determined.

Application of Bacterial Exopolymers in Metal-Scavenging Processes

Numerous industrial and mining activities produce liquid wastes containing recoverable metal ions. Biomass and biopolymers have been used to complex the dissolved metal ions to a solid phase, thereby reducing their concentrations in the bulk liquid. In recent years, reactors such as the AMT-BioClaim process, marketed by Advanced Mineral Technologies, Inc., Golden, Colorado, represent successful attempts to utilize biosorption in a technologically feasible way.

Reactor design plays a crucial role in determining the efficiency of metal recovery. The nature of the solid support will generally determine many of the reactor parameters. Since bacterial exopolymers can exist as gels, colloids, and dissolved species, they exhibit a flexibility that is somewhat unique among other biopolymers and synthetic polymers. The ability of bacterial exopolymers to exist in these different phases should maximize exposure of the binding sites to the metals dissolved in the bulk liquid phase. The flexibility of the colloid and gel states should also provide advantages over other solid supports for ease of removal from the reactor.

One of the primary considerations of the solid support is its specificity or selectivity for certain metals. There is presently a great need to identify biopolymers that demonstrate high selectivity toward metals such as Cu, Co, Ni, Zn, Cr, and Ag and minimal reactivity with cations such as Na, Ca, K, and Mg. Manipulation of the culture conditions and the genetic engineering of exopolymer-producing microorganisms should provide a useful approach to achieve this goal. Specific stereochemistry features of microbial exopolymers should soon be identified with specific genes and their products so that the desired conformations can be selected. Presumably, other parameters such as binding-site density or binding capacity could be modulated at the gene level as well.

Identification of exopolymers that exhibit very high stability constants for metals will increase the value of these bioproducts in metal removal from aqueous media. There is an increasing demand for water used in processes in the electronics industry to contain less than 1 ppb of certain metals. "Polishing" industrial water and effluents is still a major challenge to industry. The high reactivity of polysaccharides to various reagents should facilitate their chemical modification to achieve stability constants that are orders of magnitude greater than their naturally occurring analogues.

One parameter that appears to be particularly intriguing in the case of bacterial exopolymers is the possibility of creating high diffusivity of metal ions within the polymer phase by manipulating the charge properties of the various functional groups on the poly-

mer. Addition of certain anions to metal-containing wastes also appears to accelerate the rate of metal diffusion through the polymer matrix (Jang, unpublished results).

The multiple phases in which bacterial exopolymers exist offer opportunities to create more favorable flow patterns within a reactor. New flow designs should yield lower energy requirements while increasing mass transfer efficiency for metal diffusion from the bulk liquid to the surface of the biopolymer matrix (Jang, unpublished results).

The molecular volume changes that occur in exopolymers as a result of metal-ion binding in aqueous solution should facilitate separation of metal-saturated polymer from unsaturated polymer. As a polymer complexes more metal, the increased polymer density should enable the complex to migrate to a different position in a reactor than uncomplexed polymer. This characteristic as well as the plasticity of the polymer may allow for the construction of reactors that operate continuously without any downtime to replace the spent complexing agent.

It is evident from the above discussion that new and exciting prospects exist for bacterial exopolymers in metal-recovery operations. Although our current knowledge of interactions between bacterial exopolymers and metal ions is somewhat limited, the information that is available suggests that the polysaccharide fraction has the potential to form unique associations with metals that are important ecologically and economically. Presumably, research in these areas will help to advance our understanding of the role that bacterial exopolymers have in controlling the distribution of metal ions in nature.

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